Respiratory Medicine (2004) **98**, 542–550

ELSEVIER

respiratoryMEDICINE

# Multiplex PCR for the simultaneous detection of *Chlamydia pneumoniae*, *Mycoplasma pneumoniae* and *Legionella pneumophila* in community-acquired pneumonia

**Naoyuki Miyashita[a,*], Atsushi Saito[b], Shigeru Kohno[c], Keizo Yamaguchi[d], Akira Watanabe[e], Hiroshi Oda[f], Yukumasa Kazuyama[g], Toshiharu Matsushima[a], the CAP Study Group**

[a]*Division of Respiratory Diseases, Department of Internal Medicine, Kawasaki Medical School, 577 Matushima, Kurashiki City, Okayama 701-0192, Japan*
[b]*First Department of Internal Medicine, Faculty of Medicine, University of the Ryukyus, Okinawa 903-0215, Japan*
[c]*Second Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki 852-8523, Japan*
[d]*Department of Microbiology, Toho University School of Medicine, Tokyo 143-8540, Japan*
[e]*Department of Respiratory Medicine, Division of Cancer Control, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Miyagi 980-8575, Japan*
[f]*Department of Bacteriology, Faculty of Medicine, Kagoshima University, Kagoshima 890-8520, Japan*
[g]*Kitasato-Otsuka Biomedical Assay Laboratory, Kanagawa 228-8555, Japan*

Received 27 August 2003; accepted 13 November 2003

**KEYWORDS**
Multiplex PCR;
*Chlamydia pneumoniae*;
*Mycoplasma pneumoniae*;
*Legionella pneumophila*;
Community-acquired pneumonia

**Summary** A multiplex polymerase chain reaction (PCR) was developed for the simultaneous detection of *Chlamydia pneumoniae*, *Mycoplasma pneumoniae* and *Legionella pneumophila*. Oligonucleotide primers for the amplification of the DNA of these three organisms were optimized for use in combination in the same reaction. PCR products were detected by the Micro-Chip Electrophoresis Analysis System. Clinical samples were obtained from 208 community-acquired pneumonia (CAP) patients who were participants in a multicenter CAP surveillance study performed at seven medical schools and their affiliate hospitals in Japan. No significant differences in the sensitivity of each primer set were observed when tested in both the multiplex and monoplex PCR assays. Our multiplex PCR was able to reliably detect 10 copies/100 μl of each of the three pathogen DNAs. Of the panel of 208 samples, 14 of 15 *C. pneumoniae*, 10 of 10 *M. pneumoniae*, eight of eight *L. pneumophila* and 165 of 176 negative samples were correctly identified. Eleven cases who were the multiplex PCR positive and conventional method negative were observed. The PCR findings were of possible significance in at least four of these patients. Our multiplex PCR assay could potentially be used as a diagnostic and

*Tel.: +81-86-462-1111; fax: +81-86-464-1041.
*E-mail address:* nao@med.kawasaki-m.ac.jp (N. Miyashita).

0954-6111/$ - see front matter 
doi:10.1016/j.rmed.2003.11.012

epidemiological tool. Further prospective studies are needed to establish its clinical usefulness.


## Introduction

Atypical pathogens including *Chlamydia pneumoniae*, *Mycoplasma pneumoniae* and *Legionella pneumophila* are an important cause of community-acquired pneumonia (CAP). *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* are the most common pathogens in the outpatient setting and high incidences have been recorded in hospitalized patients with CAP.[1–3] *L. peumophila* has also been seen in the outpatient setting,[2,3] but this pathogen has been identified with the common organism in patients with CAP requiring the intensive care unit.[1] The incidence of infection with these atypical pathogens has been as high as 40–60% of all admitted patients, often as part of a mixed infection in Western countries.[4] In Japan, there have been some reports on the etiology of CAP among the Japanese population.[5–7] All these studies demonstrated that *C. pneumoniae* and *M. pneumoniae* are common pathogens, the third or fourth leading pathogens, and that the etiology of CAP in Japan does not differ significantly from that of Western countries.

Current methods for the identification of atypical pathogens include culturing, rapid antigen detection assays, serology and molecular techniques. Cell cultures for detection of *C. pneumoniae* require specialized laboratories and are expensive, time-consuming and labor-intensive. Since *M. pneumoniae* and *L. pneumophila* grow slowly and lack sensitivity, the clinical usefulness of cultures is limited. Rapid laboratory tests such as antigen detection or hybridization are of limited sensitivity. Serology usually requires documentation of a rise in antibody concentration from an acute phase serum sample to a convalescent phase serum sample, and thus, test results come in too late to be of relevance for the treatment of acute disease.

Currently available nucleic acid amplification (NAA) techniques, such as the polymerase chain reaction (PCR), are highly sensitive techniques for the rapid detection of nucleic acid sequences from viruses and bacteria in clinical specimens. These NAA techniques are particularly advantageous for the detection of fastidious or difficult to culture organisms such as atypical pathogens. Several conventional PCR assays have been developed and have demonstrated sensitivity and specificity equal to or even better than those of conventional microbiologic tests.[8,9] More recently, multiplex PCR assays for the simultaneous detection of different pathogens have been developed.[10–13] Here, we report on the development and evaluation of a multiplex PCR for the simultaneous detection of *C. pneumoniae, M. pneumoniae* and *L. pneumophila* in respiratory samples.

## Subjects and methods

### Origin of the samples

Nasopharyngeal swab specimens which were collected during a multicenter CAP surveillance study and stored at $-20^{\circ}C$ were used in this study. A multicenter CAP surveillance study was performed in seven medical schools and their affiliate hospitals in Japan, including the University of the Ryukyus, Kagoshima University, Nagasaki University School of Medicine, Kurume University School of Medicine, Toho University School of Medicine, Tohoku University, Kawasaki Medical School and their affiliate hospitals between December 1999 and March 2000. The diagnosis was based on clinical signs and symptoms (cough, fever, productive sputum, dyspnea, chest pain, or abnormal breath sounds) and radiographic pulmonary abnormalities that were at least segmental and were due to preexisting or other known causes. Two hundred and thirty-nine CAP patients were enrolled in this study and eventually 208 patients (17–99 years of age; 120 males and 88 females) were analyzed.

### Microbiologic laboratory tests

Blood cultures and nasopharyngeal swab specimens were obtained from 64% and 100% of the patients, respectively; when sputum was available, a Gram's stain test and a quantitative culture was obtained. Sputum data were only evaluated when the Gram's stain test revealed numerous leukocytes ($>25$ in a $100\times$ microscopic field) and few squamous epithelial cells ($<10$ in a $100\times$ microscopic field). Certain invasive methods such as bronchoscopic examination were employed to obtain specimens in some patients after full explanation of the procedures. These specimens were also used for culturing of *Legionella* spp. on buffered charcoal-yeast extract

alpha agar. Cultures for *C. pneumoniae* and *C. psittaci* were performed in cycloheximide-treated HEp-2 cells grown in a 24-well cell culture plate.[14] All specimens were passed twice. Culture confirmation was done by fluorescent-antibody staining with *C. pneumoniae* and *C. psittaci* species-specific and genus-specific monoclonal antibodies.[14]

Paired serum samples were collected at intervals of at least 4 weeks and stored at −70°C until testing. Complement fixation (CF) and passive agglutinin (PA) tests were used to detect antibodies to *M. pneumoniae*. Antibodies to *Legionella* spp. and *Coxiella burnetii* were measured by the indirect immunofluorescence test. The micro-immunofluorescence test was used for titration of IgG and IgM antibodies against *Chlamydia* spp. using formalinized elementary bodies of the *C. pneumoniae* TW-183 (purchased from the Washington Research Foundation, Seattle, WA, USA) and KKpn-15,[15] *C. trachomatis* L2/434/Bu and *C. psittaci* 6BC strains as antigens. Sera with IgM against *Chlamydia* spp. were retested after absorption with goat anti-human IgG antibody reagent (Gullsorb; Gull Laboratories, Salt Lake City, UT, USA) in order to exclude false-positive reactions. Antibodies to viruses such as influenza A and B viruses, adenovirus, respiratory syncytial virus, cytomegalovirus and parainfluenza virus types 1, 2 and 3 were measured at Kitasato-Otsuka virus assay laboratory. In addition to serology and culturing, the urinary antigen test was used for detection of *L. pneumophila* (NOW *Legionella*; Binax Inc., Portland, USA).

## Criteria for determination of microbial etiology

Bacteria were considered to be definitive causative agents when isolated from blood culture. We considered the results of sputum cultures in combination with Gram's stain findings. An organism showing heavy ($\geqq 10^7$ colony-forming units [CFU]/ml) growth of a predominant bacterium on a sputum culture or moderate ($10^{5-6}$ CFU/ml) growth of a culture with phagocytosis observed by Gram's stain was considered to be a definitive pathogen. If an organism showing moderate growth of a predominant bacterium on a sputum culture without phagocytosis was observed by Gram's stain, that specimen was judged by the medical doctor according to clinical findings and course. If *Legionella* spp. was isolated from a specimen, that specimen was considered to be a definitive pathogen even if the culture showed little growth. *L. pneumophila* was considered to be a presumptive agent when the urinary antigen test result was positive. For serologic tests, a four-fold rise in the antibody titer level between paired sera was considered definitive. *C. pneumoniae* infection was defined as IgM ≧1:32 or a four-fold rise in IgG or IgM.

## DNA extraction

A 1 ml aliquot of specimens was centrifuged at 15,000*g* for 10 min at room temperature. Most of the supernatant was discarded and 200 ml, including the pellets, were recovered and incubated with protenase K (20 μl) in AL buffer (200 μl, Qiagen) for 10 min at 56°C. Subsequently, the samples were mixed with 100% ethanol (200 μl). The DNA was extracted using the Qiagen DNA mini kit (QIA amp, Qiagen) according to the manufacturer's recommendations. Then samples were treated with ethanol three times. DNA was eluted in a final volume of 200 μl, aliquoted, and stored at −20°C before performance of the PCR.

## Multiplex PCR

Target sequences were regions of the major outer membrane protein gene for *C. pneumoniae* (CPN 25: 5′-CTC GTT GGT TTA TTC GGA GTT AAA G-3′, CPN 26: 5′-GAG AAT TGC GAT ACG TTA CAG ATC A-3′), the nucleotide sequence of the 16S rRNA for *M. pneumoniae* (MP 6: 5′-ATT GCC TTG GTA GGC CGT TAC CCC AC-3′, MP 8: 5′-CAA AGT TGA AAG GAC CTG CAA G-3′) and the major outer membrane protein porin gene for *L. pneumophila* (LPN 15: 5′-AGT GCT TTG TTT GCA GGT ACG-3′, LPN 16: 5′-CAC CAA CAT CAG TAA AAC CAT TAT AGC-3′). PCR reactions for these three pathogens were initially evaluated separately using serial dilutions of culture materials. The reactions were subsequently combined. Optimization of the multiplex assay was performed by testing different combinations of primer concentrations and magnesium concentrations, using a fixed dilution of the culture materials at about 2 log above the detection threshold of the individual assays, to establish a combination with the most comparable result. Finally, the reaction conditions of the multiplex PCR were 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 5% dimethylsufoxide, 200 μM dNTPs, 5 U AmpliTaq DNA polymerase and the presence of concentrations (0.2–0.4 μM) of three primer pairs (Applied Biosystems). The PCR was performed in a Gene Amp PCR system 9600-R (Perkin-Elmer) with the following thermal profile: 10 min at 95°C, followed by 40 cycles of 30 s at 94°C, 30 s 60°C and 60 s 72°C and one cycle for

10 min at 72°C. PCR product detection was performed with the Micro-Chip Electrophoresis Analysis System (SV1210 Cosmoeye, Hitachi).[16] The appearance of 236, 157 and 88 base pair amplification products corresponding to *C. pneumoniae*, *L. pneumophila* and *M. pneumoniae*, respectively, was considered to be a positive reaction. In each experiment, negative and positive controls for each pathogen were used. Positive controls were made with the PCR-TOPO 2.1 cloning kit (Invitrogen).

### Sensitivity and specificity of the multiplex PCR

The sensitivity of the PCR assay was determined using serial 10-fold dilutions of DNA samples from *C. pneumoniae* (ATCC VR-2282), *M. pneumoniae* (ATCC 29342) and *L. pneumophila* (ATCC 33153) with known starting concentrations. The specificity of the multiplex PCR was assessed using isolates of one of the following pathogens: *Aspergillus fumigatus*, *Aspergillus* spp., *Candida albicans*, *Candida* spp., *C. pneumoniae*, *C. trachomatis*, *C. psittaci*, *Escherichia coli*, *Haemophilus influenzae*, *Haemophilus* spp., *Klebsiella pneumoniae*, *Klebsiella* spp., *L. pneumophila*, *Legionella* spp., *M. pneumoniae*, *Mycoplasma* spp., *Staphylococcus aureus*, *Staphylococcus* spp., *Streptococcus pneumoniae*, *S. pyogenes*, *S. agalactiae*, adenovirus 1, 2, 3, 5, 7 and 11, Cytomegalovirus, Epstein Barr virus, herpes simplex virus, influenza virus A and B, parainfluenza virus 1, 2 and 3, Respiratory cyncitial virus and varicella zoster virus.

## Results

### Sensitivity and specificity of the multiplex PCR

The reproducibility of the cut-off results was determined by testing duplicates of a 10-fold serial dilution of the positive controls in 10 independent experiments. No significant differences in the sensitivity of each primer set were observed when tested in both the multiplex and monoplex PCR assays. Our multiplex PCR was able to reliably detect 10 copies/100 μl of each of the three pathogen DNAs. We also measured the amount of DNA of the PCR products in each of the three pathogens in 10 independent experiments. Based on the results from average DNA concentrations, we decided the level of semi-quantitation as follows: 10 copies/100 μl indicate +, $10^2$ copies/100 μl indicate 2+ and $10^3$ copies/100 μl indicate 3+. The specificity of the PCR reaction was considered satisfactory as no specific amplification was noted with any of the tested pathogens.

### Analysis of *C. pneumoniae*, *M. pneumoniae* and *L. pneumophila* infections using respiratory samples with CAP

Among our 208 CAP cases, the most common pathogens were *Streptococcus pneumoniae*, found in 52 cases, followed by *Haemophilus influenzae* in 38 cases, *C. pneumoniae* in 15 cases, *M. pneumoniae* in 10 cases, *L. pneumophila* in eight cases, *Staphylococcus aureus* in seven cases and *Moraxella catarrahalis* in five cases by using the conventional methods (culture, serology or antigen detection test). All 15 cases of *C. pneumoniae* demonstrated four-fold or greater rises in IgG antibody titers and five cases demonstrated positive IgM antibody titers. No culture-positive patient was observed among these cases. Of the 15 cases, *C. pneumoniae* was the only pathogen identified in nine cases (60.0%), while one or more additional etiological agents was found in six cases (40.0%). The additional pathogens were *S. pneumoniae* in two, *H. influenzae* in two, *M. catarrahalis* in two and *M. pneumoniae* in one. Among all the mixed-*C. pneumoniae* pneumonia cases, one additional agent was found in five cases and two additional agents were found in one case. All 10 cases of *M. pneumoniae* demonstrated four-fold or greater rises in CF or PA antibody titers. Of the 10 cases, one additional etiological agents was found in three cases (30.0%). The additional pathogens were *S. pneumoniae* in two and *C. pneumoniae* in one. Among eight cases of *L. pneumophila,* all cases demonstrated four-fold or greater rises in antibody titers and one culture-positive patient and two urinary antigen-positive patients were observed. Of the eight cases, one or more additional etiological agents was found in four cases. The additional pathogens were *H. influenzae* in two, *S. pneumoniae* in one, *M. catarrahalis* in one and *C. psittaci* in one. Among all the mixed-*L. pneumophila* pneumonia cases, one additional agent was found in three cases and two additional agents were found in one case.

A total of 208 CAP cases were investigated by the multiplex PCR. The multiplex PCR results were concordant with those obtained by conventional methods for the 208 cases, with positive concordant results for 31 cases (13 for *C. pneumoniae*, nine for *M. pneumoniae*, one for dual infection with *C. pneumoniae* and *M. pneumoniae* and eight for *L. pneumophila*) and negative concordant results for

**Table 1** Comparison of multiplex PCR and conventional methods for 208 patients.

| | Multiplex PCR | | | One hundred and sixty six patients negative for the three pathogens |
|---|---|---|---|---|
| | Forty-two patients for | | | |
| | *C. pneumoniae* | *M. pneumoniae* | *L. pneumophila* | |
| Patients with conventional methods positive for | | | | |
| *C. pneumoniae* | 14* | | | 1 |
| *M. pneumoniae* | | 10* | | 0 |
| *L. pneumophila* | | | 8 | 0 |
| Patients with conventional methods negative for the three pathogens | 2* | 6* | 4 | 165 |

*Including one patient with dual infection with *C. pneumoniae* and *M. pneumoniae* infection.

165 cases (Table 1). Discrepant results were observed in 12 cases. An overall agreement of 94.2% with conventional methods was obtained for the 208 cases.

The details of *C. pneumoniae*, *M. pneumoniae* and *L. pneumophila* positive cases using conventional methods and Multiplex PCR results are listed in Table 2. Of these, only one discrepant result was observed in a patient (patient No. 2) who was serology-positive for *C. pneumoniae* (four-fold rise in both IgM and IgG antibody titer) but multiplex PCR-negative. In this case, a diagnosis of *C. pneumoniae* pneumonia was made because of the serological result, the absence of other respiratory pathogens and his response to macrolide. This might suggest that the amount of DNA in this sample was below the detection limit. Dual infection with *C. pneumoniae* and *M. pneumoniae* was observed in only one patient (patient No. 15) and the multiplex PCR was also positive in both pathogens.

Among the 208 CAP cases, 11 cases who were the multiplex PCR positive and conventional method negative were observed, one for *C. pneumoniae*, five for *M. pneumoniae*, one for dual infection with *C. pneumoniae* and *M. pneumoniae* and four for *L. pneumophila* (Table 3). On the basis of bacterial cultures, clinical features and the response to antibiotics, the multiplex PCR results were considered significant in three of these 11 cases (patient No. 3, 9, and 11). In one other case (patient No. 10), another significant bacterial pathogen was isolated, but on the basis of the response to the prescribed antibiotic, the role of *L. pneumophila* could not be excluded. Seven other cases had DNA with one of the three atypical pathogens, but all responded to antibiotics not suitable for the treatment of atypical pathogens. Though the PCR results in these seven cases may seem irrelevant, they may simply indicate that in the normal host these infections are self-limiting.

## Discussion

The etiologic diagnosis of infections with atypical pathogens such as *C. pneumoniae*, *M. pneumoniae* and *L. pneumophila* still remains difficult. This is mainly due to difficulties in culturing and to the delayed results associated with conventional methods (serology and culturing), which often allow a retrospective diagnosis only. The current development of NAA techniques should offer a highly sensitive, specific and rapid diagnosis of CAP. In this study, we reported the successful development of a multiplex PCR for the simultaneous detection and differentiation of *C. pneumoniae*, *M. pneumoniae* and *L. pneumophila*.

In the experiments performed with clinical specimens, it was possible to test all specimens simultaneously for the presence of nucleic acids

**Table 2** Test results of the positive members of the evaluation panel.

| Patient no. | Multiplex PCR | | | *C. pneumoniae* | | *M. pneumoniae* | *L. pneumophila* | | |
|---|---|---|---|---|---|---|---|---|---|
| | CP | MP | LP | Serology | Culture | Serology | Serology | Culture | Urinary antigen |
| 1 | 2+ | — | — | + | — | — | — | — | — |
| 2 | — | — | — | + | — | — | — | — | — |
| 3 | 2+ | — | — | + | — | — | — | — | — |
| 4 | + | — | — | + | — | — | — | — | — |
| 5 | + | — | — | + | — | — | — | — | — |
| 6 | + | — | — | + | — | — | — | — | — |
| 7 | + | — | — | + | — | — | — | — | — |
| 8 | + | — | — | + | — | — | — | — | — |
| 9 | + | — | — | + | — | — | — | — | — |
| 10 | + | — | — | + | — | — | — | — | — |
| 11 | + | — | — | + | — | — | — | — | — |
| 12 | + | — | — | + | — | — | — | — | — |
| 13 | + | — | — | + | — | — | — | — | — |
| 14 | + | — | — | + | — | — | — | — | — |
| 15 | + | 2+ | — | + | — | + | — | — | — |
| 16 | — | 2+ | — | — | — | + | — | — | — |
| 17 | — | 2+ | — | — | — | + | — | — | — |
| 18 | — | + | — | — | — | + | — | — | — |
| 19 | — | 2+ | — | — | — | + | — | — | — |
| 20 | — | 2+ | — | — | — | + | — | — | — |
| 21 | — | 2+ | — | — | — | + | — | — | — |
| 22 | — | + | — | — | — | + | — | — | — |
| 23 | — | + | — | — | — | + | — | — | — |
| 24 | — | 2+ | — | — | — | + | — | — | — |
| 25 | — | — | + | — | — | — | + | + | + |
| 26 | — | — | + | — | — | — | + | — | + |
| 27 | — | — | + | — | — | — | + | — | — |
| 28 | — | — | + | — | — | — | + | — | — |
| 29 | — | — | + | — | — | — | + | — | — |
| 30 | — | — | + | — | — | — | + | — | — |
| 31 | — | — | + | — | — | — | + | — | — |
| 32 | — | — | + | — | — | — | + | — | — |

CP = *Chlamydia pneumoniae*; MP = *Mycoplasma pneumoniae*; LP = *Legionella pneumophila*.

from three atypical pathogens with comparatively little effort, thus indicating that this method is well-suited for use in epidemiological studies as well as for rapid microbiological studies in the clinical setting. Tong et al.[10] reported good agreement of a multiplex PCR and conventional methods using a panel of known positive and negative samples for *M. pneumoniae*, *C. pneumoniae* and *C. psittaci*. Of their panel of 53 samples, nine of 11 *M. pneumoniae*, 11 of 11 *C. pneumoniae*, six of seven *C. psittaci* and 24 of 24 negative samples were correctly identified. Welti et al.[13] also reported good agreement of a multiplex real-time PCR and serology using 38 patients with *C. pneumoniae*, *M. pneumoniae* or *L. pneumophila* respiratory tract infections. Of 19 serology-positive patients, 14 were confirmed by the multiplex real-time PCR to be infected by one of the three pathogens. All samples from serology-negative patients were negative with the multiplex real-time PCR. In our study, all but one sample from conventional method positive patients were also positive with the multiplex PCR. In addition, 165 samples from conventional method negative patients were also negative with the multiplex PCR (Table 1). Our results are consistent with these reported by Tong et al.[10] and Welti et al.[13] and indicate that the multiplex PCR assay is a useful and rapid diagnostic tool for the management of community-acquired atypical pneumonia.

Grondahl et al.[11] reported a multiplex reverse transcription-PCR protocol for detecting up to nine different respiratory agents, including *C. pneumoniae* and *M. pneumoniae*, in children. But that

**Table 3** Clinical features, bacterial culture results and treatment responses of the 11 patients who had a positive multiplex PCR result.

| Patient no. | Age (year) | Sex | Underlying conditions | Bacterial culture results | Initial antibiotic treatment | Response of initial treatment | Further treatment | Multiplex PCR resutls | Relevance and significance of PCR result |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 89 | M | Pulmonary emphysema | *S. pneumoniae* | PIPC | Yes | None | *C. pneumoniae* | No |
| 2 | 58 | M | Heart disease | *H. influenzae* | CTM FMOX | Yes | None | *C. pneumoniae* *M. pneumoniae* | No |
| 3 | 27 | F | None | Normal flora | CAM | Yes | None | *M. pneumoniae* | Yes |
| 4 | 47 | F | None | Normal flora | SBT/ABPC | Yes | None | *M. pneumoniae* | No |
| 5 | 80 | F | None | Normal flora | CLDM | Yes | None | *M. pneumoniae* | No |
| 6 | 73 | M | Old pulmonary tuberculosis | *S. pneumoniae* *M. catarrahalis* | PIPC | Yes | None | *M. pneumoniae* | No |
| 7 | 55 | M | Liver dysfunction | Normal flora | PAPM/BP | Yes | None | *M. pneumoniae* | No |
| 8 | 29 | M | None | Normal flora | CAM | No | PIPC | *L. pneumophila* | No |
| 9 | 48 | M | Heart disease | Normal flora | CAM | Yes | None | *L. pneumophila* | Yes |
| 10 | 76 | M | Bladder Ca. | *H. influenzae* | CFPM | No | IPM/CS EM Steroid | *L. pneumophila* | Possible |
| 11 | 29 | F | None | Normal flora | CAM | Yes | None | *L. pneumophila* | Yes |

PIPC = piperacillin sodium; CTM = cefotiam HCl; FMOX = flomoxef sodium; CAM = clarithromycin; SBT/ABPC = sulbactam/ampicillin; CLDM = clindamycin; PAPM/BP = panipenem/betamipron; CFPM = cefepime HCl; IPM/CS = imipenem/cilastatin; EM = erythromycin.

study reported a low detection rate for *C. pneumoniae* in contrast to other studies.[17,18] This may reflect a specimen choice problem; i.e., nasopharyngeal aspirates versus swabs, as suggested by the same authors.[19] It has been suggested that the sensitivity of PCRs differ when different types of respiratory samples are used and that the highest sensitivity and specificity to diagnose *C. pneumoniae*, *M. pneumoniae* and *L. pneumophila* could be obtained with sputum and broncho alveolar lavage.[20,21] Therefore, more studies using different types of specimens are needed to evaluate our multiplex PCR assay.

One of the characteristics of NAAs is their ability to detect non-viable organisms that have not been cleared, especially after antibiotic treatment.[22] Several studies suggest that many cases of CAP with an undetermined etiologic diagnosis are associated with previous antibiotic administration.[23,24] Thus while antibiotic treatment compromises the chance of bacterial isolation, PCR detection is not affected by the lack of viability of the pathogens and is more sensitive than cultures for the detection of atypical pathogens in respiratory specimens.[10] In contrast, prolonged and asymptomatic shedding of *C. pneumoniae* and *M. pneumoniae* has been reported.[17,18,25–30] Therefore, one should be aware that when using a sensitive PCR technique, it is possible to detect a persistent bacterial genome for a prolonged period. In this study, we also analyzed the 11 patients who were multiplex PCR positive but conventional method negative with regard to whether the episode of CAP was caused by the identified organism. Some patients (Table 3, No. 3, 9, and 11) responded to initial treatment with the appropriate antibiotic for *M. pneumoniae* and *L. pneumophila* and no other significant bacterial pathogens were isolated in these patients. So, the multiplex PCR result was considered significant in these three cases. In contrast, several patients (Table 3, No. 1, 2, 4, 5, 6, 7, and 8) were treated with inappropriate antibiotics for atypical pathogens and appeared to have a prolonged or asymptomatic infection. Overall, we believe that in at least four of 11 patients in this series, the finding of *M. pneumoniae* or *L. pneumophila* could be clinically relevant.

In conclusion, we have successfully developed a rapid, highly sensitive and specific semi-quantitative multiplex PCR that can simultaneously detect and differentiate three causative agents of atypical pneumonia. To study the usefulness of this assay in a clinical context, it is necessary to carry out prospective studies covering both epidemic and non-epidemic periods, using different types of respiratory samples.

## Acknowledgements

The authors wish to thank members of the CAP Study Group. This work was supported by Project Research Grants from Kawasaki Medical School (13-401, 14-402, 15-405A).

## References

1. Marrie TJ. Community-acquired pneumonia: epidemiology, etiology, treatment. *Infect Dis Clin North Am* 1998;**12**:723–40.
2. Wattanathum A, Chaoprasong C, Nunthapisud P, et al. Community-acquired pneumonia in southeast Asia. The microbial differences between ambulatory and hospitalized patients. *Chest* 2003;**123**:1512–9.
3. Blanquer J, Blanquer R, Borras R, et al. Etiology of community-acquired pneumonia in Valencia, Spain: a multicenter prospective study. *Thorax* 1991;**46**:508–11.
4. The American Thoracic Society. Guidelines for the management of adults with community-acquired pneumonia. Diagnosis, assessment of severity, antimicrobial therapy, and prevention. *Am J Respir Crit Care Med* 2001; **163**:1730–54.
5. Ishida T, Hashimoto T, Arita M, Ito I, Osawa M. Etiology of community-acquired pneumonia in hospitalized patients: a 3-year prospective study in Japan. *Chest* 1998;**114**:1588–93.
6. Ishida T, Hashimoto T, Arita M, et al. Evaluation of community-acquired pneumonia guidelines of Japanese respiratory society: differentiation of atypical pneumonia and bacterial pneumonia. *Nihon Kokyuki Gakkai Zasshi* 2002;**40**:929–35 (in Japanese).
7. Miyashita N, Fukano H, Niki Y, Matsushima T, Okimoto N. Etiology of community-acquired pneumonia requiring hospitalization in Japan. *Chest* 2001;**119**:1295–6.
8. Boman J, Gaydos CA, Quinn TC. Molecular diagnosis of *Chlamydia pneumoniae* infection. *J Clin Microbiol* 1999;**37**:3791–9.
9. Dorigo-Zetsma JW, Zaat SA, Wertheim-van Dillen PM, et al. Comparison of PCR, culture, and serological tests for diagnosis of *Mycoplasma pneumoniae* respiratory tract infection in children. *J Clin Microbiol* 1999;**37**:14–7.
10. Tong CYW, Donnelly C, Harvey G, Sillis M. Multiplex polymerase chain reaction for the simultaneous detection of *Mycoplasma pneumoniae*, *Chlamydia pneumoniae* and *Chlamydia psittaci* in respiratory samples. *J Clin Pathol* 1999;**52**:257–63.
11. Grondahl B, Puppe W, Hoppe A, Kuhne I, Weigl JAI, Schmitt H-J. Rapid identification of nine microorganisms causing acute respiratory tract infections by single-tube multiplex reverse transcription-PCR: feasibility study. *J Clin Microbiol* 1999;**37**:1–7.
12. Corsaro D, Valassina M, Venditti D, Venard V, Faou AL, Valensin PE. Multiplex PCR for rapid and differential diagnosis of *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* in respiratory infections. *Diagn Microbiol Infect Dis* 1999;**35**:105–8.
13. Welti M, Jaton K, Altwegg M, Sahli R, Wenger A, Bille J. Development of a multiplex real-time PCR assay to detect *Chlamydia pneumoniae, Legionella pneumophila* and *Mycoplasma pneumoniae* in respiratory tract secretions. *Diagn Microbiol Infect Dis* 2003;**45**:85–95.

14. Miyashita N, Fukano H, Okimoto N, et al. Clinical presentation of community-acquired *Chlamydia pneumoniae* pneumonia in adults. *Chest* 2002;**121**:1776–81.
15. Miyashita N, Kubota Y, Kimura M, et al. Characterization of *Chlamydia pneumoniae* strain isolated from a 57-year-old man. *Microbiol Immunol* 1994;**38**:857–64.
16. Lahiff S, Glennon M, O'Brien L, et al. Species-specific PCR for the identification of ovine, porcone and chicken species in meta and bone meal (MBM). *Mol Cell Probes* 2001;**15**:27–35.
17. Emre U, Roblin PM, Gelling M, et al. The association of *Chlamydia pneumoniae* infection and reactive airway disease in children. *Arch Pediatr Adolesc Med* 1994;**148**:727–31.
18. Gaydos CA, Roblin PM, Hammerschlag MR, et al. Diagnostic utility of PCR-enzyme immunoassay, culture, and serology for detection of *Chlamydia pneumoniae* in symptomatic and asymptomatic patients. *J Clin Microbiol* 1994;**32**:903–5.
19. Ieven M, Goossens H. Relevance of nucleic acid amplification techniques for diagnosis of respiratory tract infections in the clinical laboratory. *Clin Microbiol Rev* 1997;**10**:242–56.
20. Boman J, Allard A, Persson K, Lundborg M, Juto P, Wadell G. Rapid diagnosis of respiratory *Chlamydia pneumoniae* infection by nested touchdown polymerase chain reaction compared with culture and antigen detection by EIA. *J Infect Dis* 1997;**175**:1523–6.
21. Waites KB, Lanier Thacker W, Talkington DF. The value of culture and serology for detection of *Mycoplasma pneumoniae* infections in the clinical laboratory in the age of molecular diagnostics. *Clin Microbiol Newslett* 2001;**23**:123–9.
22. Menendez R, Cordoba J, de La CP, et al. Value of the PCR assay in noninvasive respiratory samples for diagnosis of community-acquired pneumonia. *Am J Respir Crit Care Med* 1999;**159**:1868–73.
23. Marrie TJ, Peeling RW, Fine MJ, Singer DE, Coley CM, Kapoor WN. Ambulatory patients with community-acquired pneumonia: the frequency of atypical agents and clinical course. *Am J Med* 1996;**101**:508–15.
24. Sopena N, Sabria M, Pedro-Botet ML, et al. Prospective study of community-acquired pneumonia of bacterial etiology in adults. *Eur J Clin Microbiol Infect Dis* 1999;**18**:852–8.
25. Gnarpe J, Gnarpe H, Sundelof B. Epidemic prevalence of *Chlamydia pneumoniae* in subjectively healthy persons. *Scand J Infect Dis* 1991;**23**:387–8.
26. Block SL, Hammerschlag MR, Hedrick J, et al. *Chlamydia pneumoniae* in acute otitis media. *Pediatr Infect Dis J* 1997;**16**:858–62.
27. Hyman CL, Roblin PM, Gaydos CA, Quinn TC, Schachter J, Hammerschlag MR. Prevalence of asymptomatic nasopharyngeal carriage of *Chlamydia pneumoniae* in subjectively healthy adults: assessment by polymerase chain reaction-enzyme immunoassay and culture. *Clin Infect Dis* 1995;**20**:1174–8.
28. Miyashita N, Niki Y, Nakajima M, Fukano H, Matsushima T. Prevalence of asymptomatic infection with *Chlamydia pneumoniae* in subjectively healthy adults. *Chest* 2001;**119**:1416–9.
29. Gnarpe J, Lundback A, Sundelof B, et al. Prevalence of *Mycoplasma pneumoniae* in subjectively healthy individuals. *Scand J Infect Dis* 1992;**24**:161–4.
30. Foy HM, Grayston JT, Kenny GE, et al. Epidemiolpgy of *Mycoplasma pneumoniae* infection in families. *JAMA* 1996;**197**:859–66.